AN ADAPTIVE PEROXIDATION BY STREPTOCOCCUS FAECALIS

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Among the facultative anaerobes the lactic-acid-forming group of bacteria is notable for its failure to produce catalase. It is somewhat paradoxical that this enzyme should be lacking in these cells whose respiration proceeds through flavin carriers and ends in H_2O_2. The accumulation of H_2O_2, which is obviously opposed to the interests of the cells, occurs in certain species of the genera *Streptococcus*, *Lactobacillus*, and *Diplococcus* (McLeod and co-workers, 1922, 1923).

McLeod and Govenlock (1921) demonstrated that *Streptococcus faecalis* could grow to a notable degree in the presence of peroxide-forming bacteria. Farrell (1935) found that peroxide was formed by *S. faecalis*. Greisen and Gunsalus (1943) mentioned that an enterococcus they tested accumulated H_2O_2 upon the oxidation of alcohol. Gunsalus and Umbreit (1945) showed H_2O_2 accumulation as a result of glycerol oxidation by *S. faecalis* F24. Accounts of other species of lactic-acid-producing organisms with reference to H_2O_2 formation are numerous, but it is not our purpose to engage in a general discussion of the subject.

The relation of *S. faecalis* to H_2O_2 production reported in most of the references cited seemed to be at odds with an observation made many times in this laboratory, namely, that certain strains of *S. faecalis* produce more cells under aerobic than under anaerobic conditions. The work reported in this paper stemmed from an investigation into the manner of aerobic growth of *S. faecalis*. In essence, evidence is presented of the adaptive formation by *S. faecalis* B33A, in an oxygen environment, of a peroxidase type mechanism. No H_2O_2 is demonstrable in cultures so grown. Greater numbers of cells are formed in shallow than in deep layers of medium. If the cells are grown in the absence of oxygen and subsequently placed on an oxidizable substrate under aerobic conditions, an accumulation of H_2O_2 soon halts the reaction. The shallow-grown and deep-grown cells have also been shown to differ in certain other respects in their metabolism under oxygen.

METHODS AND RESULTS

Culture. The culture employed during the principal part of the work was *S. faecalis* B33A. The physiology and nutrition of this strain have been thoroughly studied, and it conforms to the established characteristics of the species presented by Sherman (1937) and extended in recent years by various workers. In addition, this organism belongs to a particular strain within the species that grows with special vigor and can be distinguished from certain other strains by the speed and abundance of its growth. Gunsalus (1947) drew atten-

tion to the fact that strains of this nature ferment glycerol anaerobically and demonstrate a more diverse use of substrates than do the slower-growing strains that ferment glycerol under aerobic conditions only.

Influence of oxygen on growth. It was observed that *S. faecalis* B33A grows to a greater population in highly aerated (shaken) culture than in deep still culture. The medium used to grow the cells in all the experiments reported was ordinary beef infusion broth containing 30 per cent infusion, 1 per cent tryptone, 0.2 per cent K_2HPO_4, and 0.1 or 0.15 per cent glucose. More growth was secured when proteose peptone was substituted for tryptone, and cells for a few of the later experiments were grown in a medium of this composition. The initial pH of the media was 6.8 to 7.0.

Deep cultures were prepared by inoculating 100 ml of beef infusion broth in a 125-ml Erlenmeyer flask with 1 ml of an 18-hour test tube culture. Shallow cultures for aeration were prepared by inoculating the same volume of medium in a 4-liter Erlenmeyer flask, in which the medium was spread in a thin layer of not more than 2 or 3 mm in depth. This culture was placed on a shaker during growth. Both cultures were incubated at 34 C for 18 hours, following which 10-ml portions of each were examined with appropriate blanks at 850 millimicrons in a "kromatrol" photometer.

The shaker-grown cells always yielded a greater crop than did the deep-grown cells, a result not to be expected commonly in the genus *Streptococcus*, particularly in view of the references cited pertaining to peroxide accumulation. According to the average of 12 experiments, 30 per cent more cells were obtained on the shaker than in deep culture under the conditions outlined. Experiments in which cells were shaken under air and under nitrogen showed an average of 36 per cent more cells under air, all other conditions being identical. The analysis of the spent media for remaining glucose by the method of Kingsley and Reingold (1949) showed that the increased cell crop under air was obtained at no extra expenditure of glucose. It follows that the aerobic cells secured more energy per unit of substrate than did the deep-grown cells.

Whereas M/1,000 KCN incorporated in the medium inhibited neither the shallow nor the deep cells, M/100 iodoacetate prevented all growth.

Oxidation of glucose. Cells grown in deep and shallow layers of beef infusion broth were centrifuged, washed once with distilled water, and resuspended in 5 ml of distilled water; a dilution of the shallow-grown cells based on turbidimetric measurements was made to equalize the cell concentration of the two batches. Cell suspensions prepared in this way were used in the manometric work described hereafter. Manometric experiments were conducted along the general lines described by Umbreit, Burris, and Stauffer (1949). Cell concentrations in most experiments were adjusted to provide 3.4 mg of cells (dry weight) per cup in 1 ml of water. In the main compartment with the cells were 1 ml of phosphate buffer of pH 6.9 and water to a total cup volume of 3 ml. The side arm contained 0.5 ml of M/10 glucose and the center well contained 0.15 ml of 10 per cent KOH. Experiments were conducted at 38 C. The endogenous activity of both types of cells was negligible.

Figure 1 shows the results of the oxidation of glucose by shallow-grown and deep-grown cells of *S. faecalis* B33A. The cells grown in a shallow layer oxidize glucose rapidly, at a constant rate of oxygen consumption and with no accumulation of H_2O_2. The cells grown in deep culture oxidize glucose at first rapidly, then at a decreasing rate of oxygen consumption, and with an accumulation of H_2O_2. The test for H_2O_2 was made quantitatively according to the method of Main and Shinn (1939), and generally about 50 to 60 μg of H_2O_2 per cup caused oxygen uptake to cease. The addition of NaCN to a M/1,000 final concentration in the Warburg cup exerted no appreciable influence on glucose oxidation by either shallow-grown or deep-grown cells. The addition of M/100 cyanide slowed the reaction to some extent but other results remained the same. As in

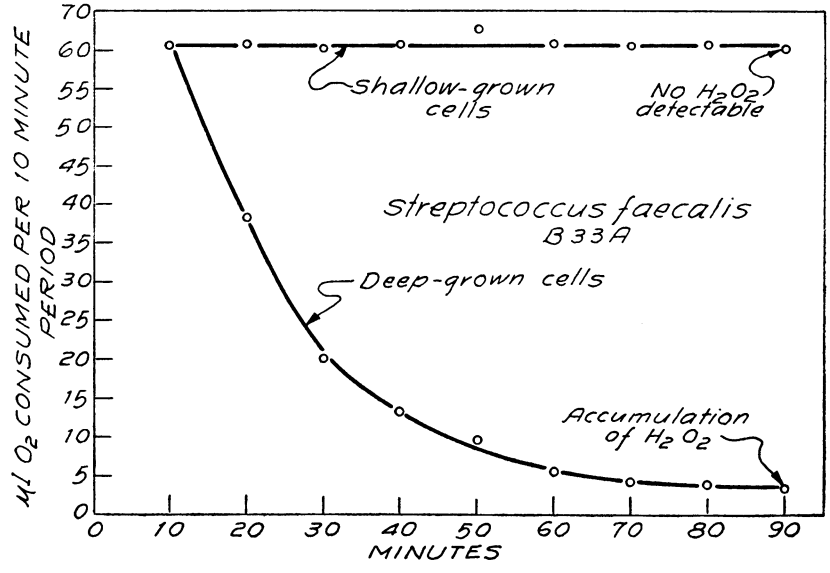

Figure 1. The oxidation of glucose by shallow-grown and deep-grown cells of *S. faecalis* B33A in relation to H_2O_2 production.

the absence of cyanide, peroxide accumulated only in the cup containing the deep-grown cells. This points to the fact that the device in the shallow-grown cells that is responsible for either the oxidation of glucose without peroxide production or the destruction of peroxide is not a hematin-containing enzyme.

In experiments in which cyanide was used in the cup, cyanide was included in the KOH in the center well.

The behavior of deep-grown cells protected against peroxide by one drop of a 1/10,000 dilution of crystalline catalase[1] closely parallels that of the shallow-grown cells in the rate of oxygen consumption. This indicates that the limiting factor in the oxidation of glucose by the deep-grown cells is H_2O_2 accumulation, and the action of catalase in removing H_2O_2 gives the same over-all effect as

[1] We are indebted to Dr. J. B. Sumner for this catalase.

does the peroxidaselike action of the shallow-grown cells. If the fact is taken into account that catalase is returning one-half of all the oxygen that appears in peroxide to the reservoir in the cup, the implication would be that the deep cells, as they are unable to use peroxide and if not interrupted by peroxide accumulation, would take up oxygen at twice the rate of the shallow cells. This is borne out by repeated observations during this work that, just after the tipping in of the substrate, the oxygen uptake of the deep cells approximated twice that of the shallow cells. With unprotected cells, peroxide accumulation rapidly alters this relationship, as would be expected.

On the addition of 0.5 ml of neutralized M/10 pyruvate to the cups containing the deep-grown cells, the rate of oxygen consumption is the greatest, approxi-

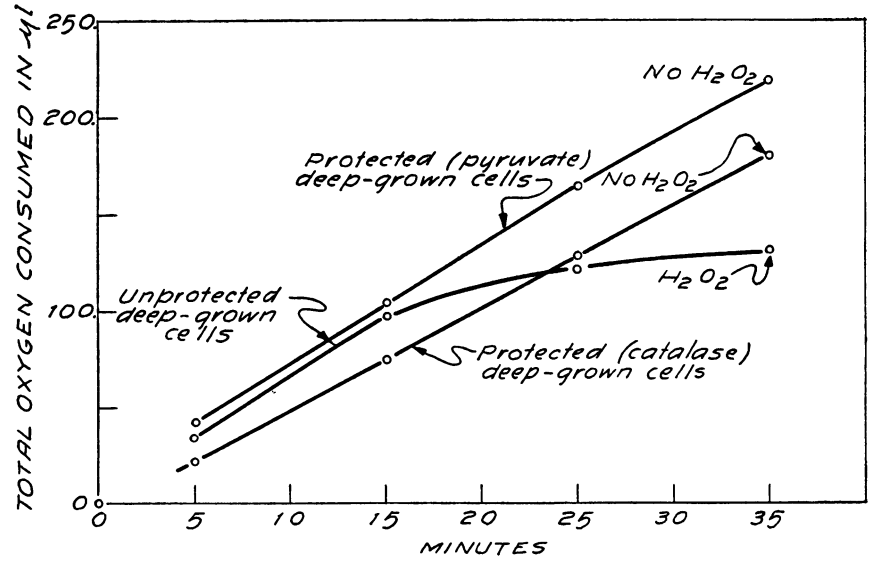

Figure 2. The relationship between total oxygen consumed in the oxidation of glucose by deep-grown cells of *S. faecalis* B33A, unprotected, protected by catalase, and protected by pyruvate.

mating that of the initial rate of the unprotected cells before the influence of the peroxide is felt. Corrections for pyruvate oxidation have been applied. These various relationships are shown in figure 2.

Oxidation of glycerol. Glycerol is oxidized at a more rapid rate by the shallow-grown cells than is glucose. No peroxide accumulates. The deep-grown cells on the other hand, oxidize glycerol at a decreasing rate and with peroxide accumulation. In general, the results on glycerol are essentially the same as those on glucose, as shown in figure 1.

Disappearance of added peroxide. Cells were prepared and brought to equal turbidity in the same manner as in the previously described manometric experiments. A 0.5-ml portion of the suspension was incubated at room temperature with 0.5 ml (34 μg) of H_2O_2, 0.5 ml of M/10 substrate, and 0.5 ml H_2O for 30

minutes, after which the cells were centrifuged and the clear supernatant liquid was examined for residual H_2O_2.

H_2O_2 added to the shallow-grown cells in small amounts disappears rapidly in the presence of glucose and glycerol and more slowly in the presence of lactate. All of these substrates can be oxidized by *S. faecalis* B33A, the rate on lactate being comparatively low. Ethyl alcohol cannot be oxidized by these cells nor will it serve as a substrate to demonstrate the disappearance of added peroxide.

The deep-grown cells reveal a very limited ability to use added peroxide in the presence of glucose, but not in the presence of glycerol. This limited ability

TABLE 1

The disappearance of H_2O_2 added to shallow-grown and deep-grown cells of S. faecalis B33A in the presence of various substrates

SHALLOW-GROWN CELLS	% H_2O_2 USED	DEEP-GROWN CELLS	% H_2O_2 USED
(1) Cells, boiled H_2O_2 Glucose	0	(1) Cells, boiled H_2O_2 Glucose	0
(2) Cells H_2O_2	0	(2) Cells H_2O_2	0
(3) Cells H_2O_2 Glucose	>95	(3) Cells H_2O_2 Glucose	12
(4) Cells H_2O_2 Glycerol	>95	(4) Cells H_2O_2 Glycerol	0
(5) Cells H_2O_2 Lactate	53		
(6) Cells H_2O_2 Ethyl alcohol	0		

of the deep-grown cells to use added peroxide is in line with observations on the oxidation of glucose in the Warburg cup, in which usually the consumption of oxygen does not entirely cease but proceeds at a fraction of the original level as though a partial adjustment to peroxide had been made. These effects may be associated with what appears to be a very rapid synthesis of the protective mechanism stimulated by contact of the cells with oxygen under suitable conditions. Table 1 contains a summary of the results of these experiments.

Utilization of oxygen and glucose. In the absence of detectable peroxide resulting from glucose oxidation by the shallow-grown cells, it seems reasonable either that peroxide was actually formed and was immediately destroyed or that a

"nascent" peroxide on enzyme surfaces was immediately redirected before the peroxide molecule was actually formed. It was our opinion at this point that, through the intervention of the adaptive device, the oxygen so occurring was "reused" in the oxidation of an unknown compound or possibly several unknown compounds, and the over-all effect is that the shallow-grown cells draw less total oxygen from the supply than do the deep-grown cells that lack this mechanism. With this possibility in mind experiments were undertaken to show the relationship between oxygen consumed and glucose used by each of the two types of cells. Oxygen consumption was measured directly and glucose consumption was calculated by difference.

Washed cells were resuspended in water, adjusted to a standard turbidity (equivalent for *S. faecalis* B33A to a dry weight of 3.4 mg per 1 ml), and 1 ml of this suspension, 0.5 ml of distilled water, and 1 ml of phosphate buffer at pH 6.9 were contained in the main compartment of the cup. The side arm contained 0.5 ml of M/10 glucose. The center well contained KOH. The oxygen consumed by deep-grown cells was measured with cells unprotected against peroxide, cells protected by catalase, and cells protected by pyruvate.

With active cells, an atmosphere of air in the cup did not supply sufficient oxygen to allow the cells to oxidize at full capacity. The replacement of air by an oxygen atmosphere largely rectified this trouble.

Following the completion of manometric measurements, the glucose test was made by transferring a 2-ml portion of the cup contents to a centrifuge tube containing 0.2 ml of trichloracetic acid (100 g in 100 ml of H_2O) to stop the reaction. After standing at room temperature for 15 minutes the cells were spun down, and 1 ml of the supernatant liquid was removed for glucose analysis. The zero time glucose was ascertained by preparing similar cups in which 0.3 ml of trichloracetic acid were present in the main compartment of the cup during the experiment. The results of this series of tests are given in table 2.

Shallow-grown cells use about 1.25 μM of glucose for each μM of O_2 consumed. The deep-grown cells, in contrast, use about 0.80 μM of glucose for each μM of O_2 expended. This value for the deep-grown cells is an average of the values from cells unprotected against peroxide and cells protected by pyruvate. These cells have in common the situation that oxygen that appears in peroxide remains unavailable to the cell for further oxidation. If one doubles the μM of O_2 consumed in the cup with added catalase, the total oxygen consumption becomes 27.4 μM and the glucose:oxygen ratio becomes 0.80, a value that agrees precisely with that obtained under the conditions described above.

Upon comparing the glucose:oxygen ratios for the shallow-grown and deep-grown cells, it is evident that the deep cells consume 1.56 times more oxygen than do the shallow cells during the dissimilation of a unit of glucose. This ratio approaches a theoretical value of 2, which would be expected here all other things being equal. The breakdown of a substrate such as glucose is relatively complex, and oxverse reactions undoubtedly occur that upset a theoretical balance, so that the lack of complete agreement in these figures is not surprising.

The fact is established, however, by these data that the shallow-grown cells accomplish considerably more oxidation with the oxygen consumed than do the deep-grown cells.

Sufficient evidence is at hand entirely to discount the possibility that the enzyme involved in these reactions is catalase. Washed cells in a Warburg cup

TABLE 2

The relation of oxygen to glucose used by shallow-grown and deep-grown cells of S. faecalis B33A

CUP CONTENTS	μM O_2	μM GLUCOSE	$\frac{\mu M \text{ GLUCOSE}}{\mu M\ O_2}$
Shallow-grown cells; air atmosphere			
(1)	20.4	28.7	1.4
(2)	21.9	34.5	1.6
(3)	15.3	15.6	1.0
(4)	17.4	23.6	1.4
(5)	19.8	25.8	1.3
(6)	19.1	25.0	1.3
			Avg 1.3
Shallow-grown cells; oxygen atmosphere			
(1)	10.8	12.3	1.1
(2)	10.8	13.3	1.2
(3)	10.5	12.3	1.2
			Avg 1.2
Deep-grown cells; oxygen atmosphere			
(1)	10.0	6.7	0.7
(2)	10.5	10.2	1.0
			Avg 0.8
Deep-grown cells; oxygen atmosphere; added catalase	13.7	21.9	1.6
Deep-grown cells; oxygen atphere; added pyruvate	9.9	7.5	0.8

under nitrogen release no gas from added H_2O_2. Peroxide does not disappear under the influence of cells except in the presence of an oxidizable substrate. The oxidation of glucose proceeds in the presence of cyanide without peroxide accumulation. These effects are contrary to those expected were catalase involved. Likewise, the ordinary peroxidase (horse-radish type) is cyanide-sensitive and does not appear to be concerned here.

DISCUSSION

The reaction reported here concerning the adaptation of *S. faecalis* B33A to oxygen, by its failure to accumulate H_2O_2, is a clear example of a Pasteur effect in the broad sense of the term employed by Burk (1939). The acquisition of this mechanism not only protects the cells from peroxide but may turn the presence of peroxide to an advantage in further oxidations.

The adaptation is demonstrable in young cultures. A 5-hour culture of *S. faecalis* 10Cl grown in shallow and deep layer showed an active peroxidaselike mechanism in the former cells, which oxidize glucose without peroxide accumulation. Examination of the records of comparable manometric experiments makes it apparent that a small number of cells, oxidizing glucose at a relatively slow rate, continue to oxidize glucose slowly, whereas a larger number of cells oxidize glucose with a rate that drops precipitously and comes to a complete standstill in some cases. This suggests that where peroxide is produced rather slowly as in newly inoculated cultures, or by small numbers of mature cells oxidizing glucose, the cells adapt to prevent peroxide accumulation.

The occurrence of a similar, if not identical, peroxidase type mechanism was described by Greisen and Gunsalus (1943, 1944) and appears to be constitutive in the strain of *Streptococcus mastitidis* they used. This organism oxidizes 1 mole of ethyl alcohol to acetic acid with 1 mole of oxygen and without the accumulation of peroxide. Whereas the extent of exposure to air of the culture during growth is not indicated in their report, it is likely that the conditions approached those of our deep-grown cells. This peroxidase type mechanism is not sensitive to cyanide, is heat-sensitive, and functions to utilize added peroxide in the presence of ethyl alcohol and to a lesser extent in the absence of alcohol.

From our experience with the adaptation and from the data given in the preceding reference, it seems likely that the reactions concerned with the peroxide utilization are of the same nature as those of the hematin-containing peroxidase.

(1) $A \cdot H_2 + O_2 \rightarrow H_2O_2 + A$
(Oxidizable substrate)

(2) $A \rightarrow B \cdot H_2$
(Intermediate oxidizable product)

(3) $B \cdot H_2 + H_2O_2 \rightarrow B + 2H_2O$
(or $A \cdot H_2 + H_2O_2 \rightarrow A + 2H_2O$)

The reports of Douglas (1947) and Hardin (1950) give evidence of peroxidase type mechanisms in *Lactobacillus brevis* and *Lactobacillus casei*. The evidence we have presented should be corroborated by studies with cell-free enzyme preparations. These studies might well show the precise substrates that are affected by this supplementary oxidizing mechanism and provide more detailed information concerning the nature of the enzyme(s).

SUMMARY

Cultures of *Streptococcus faecalis* B33A grown with free access to air produce about 30 per cent more cells for an equal expenditure of glucose than do deep-grown cultures. The shallow-grown cells oxidize glucose and glycerol at a con-

stant rate and without the accumulation of peroxide. Deep-grown cells in these circumstances accumulate peroxide to such an extent that the oxidation ceases. Shallow-grown cells rapidly utilize peroxide, added in small amounts, in the presence of oxidizable substrates. With free access to oxygen, about 1.3 μM of glucose are dissimilated per μM of oxygen consumed by the shallow-grown cells, whereas deep-grown cells use about 0.8 μM of glucose per μM of oxygen consumed. The mechanism in the shallow-grown cells that is concerned in these reactions appears to be a peroxidaselike reaction that supplements other oxidizing mechanisms of the cell.

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